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Contributions of molecular and cranial specializations for sensory adaptation in the subterranean blind mole rats (*Nannospalax xanthodon*)

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Abstract

The blind mole rats which have adapted excellently to subterranean life, exhibit remarkable navigation abilities despite structural regression in their visual systems and decline in auditory functions. This study investigates the sensory capabilities enabling blind mole rat possible adaptation to dark environments through gene expression analyses, evolutionary sequence comparisons, and cranial morphometric examinations. Comparative analyses reveal a significant reduction in the expression of genes related to vision (*Pax6*, *Impg*) and hearing (*Coch*, *Necap1*) in subterranean blind mole rat *Nannospalax xanthodon* compared to surface dwelling *Rattus rattus*. In contrast, genes associated with sound perception (*Foxp2*, *Bmp7*, *Kcnq4*, *Tmc1*) show a significant increase in expression. These findings indicate that sensory losses are aligned with the classical model observed in species that compensate for sensory deficits through echolocation. Remarkably, the gene expression profiles of *N. xanthodon* show similarities to those of mammals with highly developed echolocation abilities, revealing common molecular patterns between seismic and ultrasonic echolocation. Phylogenetic analyses of echolocation related genes show that blind mole rats share specific amino acid motifs with certain echolocating bat and whale species. Notably, positive selection signal observed in the *Kcnq4* gene suggest that this gene may play a critical role in seismic vibration detection. Cranial morphometry studies support the notion that *N. xanthodon* possess not only putative

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morphological adaptations specific to their digging lifestyle but also specialized anatomical structures for detecting seismic vibrations. This study provides contributions to understanding the sensory adaptation of subterranean mammals and evaluates blind mole rats in this context for the first time.

Keywords
Dark life, echolocation, gene expression, morphometry, phylogenetic analysis, seismic vibration

Introduction

It has been suggested that the visual and auditory adaptations acquired by nocturnal mammals to facilitate activity in the dark gave them a survival advantage over diurnal lineages that re-emerged during the Cenozoic era (Wu et al. 2017; Yan et al. 2020). Genomic and phylogenetic analyses based on the photoreceptive opsin gene family in mammals indicate that the sensory adaptation to nocturnal activity occurred approximately 215.5 million years ago (mya) (Borges et al. 2018). Increasing aridity, colder climates, and climatic changes associated with continentalization during the Cenozoic facilitated the rapid dispersal of many unrelated mammals into subterranean environments across all continents over more than 50 million years (Nevo 2022). Fossil and molecular evidence show that members of the rodent family Spalacidae, which includes extant blind mole rats, originated from a sighted murid ancestor 30 million years ago and became isolated underground (Nevo 1999). During this time, many aspects of visual anatomy and function were selectively abandoned, while photoreceptive elements necessary for regulating circadian rhythms (light/dark cycles), such as photoreceptor cells, and retinal and circadian genes, were preserved (Avivi et al. 2007; Nevo 2013). Among the 259 pseudogenes identified in the genome of the blind mole rat (*Nannospalax galili*), 22 were found to be associated with the visual system (Fang et al. 2014a). Moreover, extensive regression of lens, retinal, and eye-specific developmental genes has been investigated in blind mole rats and other subterranean species, strongly supporting previous data on visual regression (Partha et al. 2017). Subterranean environments encompass a wide range of habitats, from natural cavities such as caves to shallow subterranean spaces and groundwater systems. These systems are typically characterized by the absence of light (aphotic conditions), relatively stable microclimatic parameters such as temperature and humidity, and, in most cases, nutrient scarcity (oligotrophic conditions). The lack of light, which is the most distinctive environmental feature of these habitats, often leads to a reduction or complete loss of vision in organisms. This is commonly reflected through troglomorphic adaptations, such as the regression of eyes and loss of pigmentation (Moldovan et al. 2018; Culver and Pipan 2019). However, the deep burrow systems excavated by mole rats differ from this general definition of subterranean habitats in certain respects, offering a uniquely constructed environment that is actively shaped by the animals themselves. Although these subterranean burrows provide more stable microclimatic conditions compared to surface environments, environmental parameters such as oxygen and carbon dioxide concentrations can vary

substantially depending on burrowing behavior, soil structure, and depth (Begall et al. 2007). It has been suggested that these environmental pressures may drive not only the regression of the visual system in mole rats, but also the enhancement of other sensory modalities through compensatory adaptations (Zheng et al. 2022).

In blind mole rats (*Nannospalax ehrenbergi*) the central visual pathways, which serve form and motion perception in sighted mammals, are taken over by the auditory system, and the occipital cortex, while structurally similar, is functionally activated by auditory stimuli (Heil et al. 1991; Necker et al. 1992). Subsequent detailed histological and electrophysiological studies have raised questions about whether the visual cortex can be divided into distinct functional areas for different sensory modalities and whether it is multisensory (Bronchti et al. 2002). Unique structural features of the auditory system and the histological structure of the cochlea in blind mole rats suggest that this specialization has a significant impact on hearing capacity, acoustic communication, and orientation underground (Bruns et al. 1988; Balcioglu et al. 2021). Despite their sensitivity to light, the eyes of *Nannospalax* are structurally intact but functionally completely blind (Avivi et al. 2004). For this species, which feeds on underground plant roots in the absence of light, locating and storing food, protecting against predators, detecting physical changes in their tunnels, and communicating with individuals in neighboring tunnels are significant challenges. Researchers have explained that blind mole rats cope with these challenges by developing extraordinary sensory modalities, such as magnetoreception, vision, hearing, somatosensation, and olfaction (Kashash et al. 2022). However, it remains unclear whether they primarily use their auditory system, by pressing their lower jaws against tunnel walls to detect vibrations, or their somatosensory pathway, through mechanoreceptors in their claws, to perceive distant objects (Mason and Wenger 2019).

Echolocation is a specialized communication method based on sound production and the reception of returning echoes, allowing animals to gather information about their environment. Although the mechanism by which *Nannospalax* detects underground vibrations is debated, their extraordinary echo-location system, where the sender and receiver are the same organism, has been described as a form of echolocation (Kimchi et al. 2005). For animals using this method, detecting ultrasonic signals is not a prerequisite; rather, it is the ability to determine the location, direction, or speed of approaching obstacles or dangers by producing signals and analyzing returning echoes. The structure of echolocation signals, differences in peak spectra, environmental wavelength and sound speed, habitat, and prey parameters, and specialized hearing organs have all evolved to shape this ability (Ketten et al. 2012; Ketten et al. 2021). Although the navigational abilities of blind mole rats have been successfully demonstrated in behavioral studies, they are not considered full echolocators due to their specialization in detecting and transmitting low-frequency signals (Rado et al. 1987; Heth 1991; Kimchi and Terkel 2003; Kimchi et al. 2005). Vibrations produced by blind mole rats tapping their heads against tunnel walls have been measured at 100–200 Hz. Their vocal repertoires, difficult to detect in a closed system, are not fully understood but vary depending on the threat, captivity, and species (Nevo et al. 1987; Kashash et al. 2022).

However, some studies have recorded sounds in the frequency range of 8–16 kHz for *N. ehrenbergi*, while for *N. leucodon*, sounds initially starting at 9.8 kHz and decreasing to 2–3 kHz with attenuation up to 50 kHz have been observed both underground and on the surface when threatened (Burda 2006; Zidarova and Pandourski 2019). Due to the structure of underground tunnels, low frequencies (approximately 0.5–1 kHz) are generally more effectively propagated. Compared to rats, the hearing range of blind mole rats is not restricted to eight octaves but is specifically sensitive to low frequencies, which is an auditory adaptation to the stethoscope effect of the subterranean realm (Lange et al. 2007; Burda 2021).

The use of different organs by bats and dolphins to produce and emit sound has not prevented the development of similar sound signal detection and processing systems (Parker et al. 2013). The echolocation system, which enables the ability to locate objects using echoes, is studied as an example of genetic and functional convergence among distantly related animal groups. Research explaining the genetic basis of the echolocation system has revealed that genes involved in critical functions such as hearing and cochlear amplification, including *Prestin* (*SLC26A5*), *Kcnq4* (voltage-gated KQT-like subfamily Q-4), *Cdh23* (Cadherin-23), *Pcdh15* (Protocadherin-15), *Otof* (Otoferlin), *Tmc1* (Transmembrane channel-like 1), and *Pjvk* (Deafness, autosomal recessive-59), exhibit parallel evolution due to convergent amino acid changes in both bats and toothed whales. Additionally, positive selection pressures have influenced these genes, driving the evolution of echolocation (Li et al. 2008; Liu et al. 2010; Davies et al. 2012; Liu et al. 2012; Shen et al. 2012; Parker et al. 2013). While echolocating bats and toothed whales use ultrasonic frequencies to detect small objects, some cave-dwelling birds (such as *Aerodramus* swiftlets, pygmy swiftlets, and oilbirds), known to use echolocation, produce lower-frequency click calls. In these species, evolutionary changes in genes associated with echolocation are less pronounced compared to bats, though some convergence signals have been detected (Sadanandan et al. 2023).

Behavioral evidence suggesting echolocation abilities in the common shrew (*Sorex araneus*), a nocturnal insectivore with poor vision, and the soft-furred tree mouse (*Typhlomys*), a nearly blind semi-arboreal rodent, is molecularly supported by the detection of convergent amino acid changes in hearing genes associated with echolocation (Chai et al. 2020; He et al. 2021; Jones 2021). In addition to key genes for molecular convergence in echolocating species, *Foxp2* (Forkhead Box P2), which supports sensorimotor coordination in bats, and *Bmp7* (Bone Morphogenetic Protein 7), associated with tonotopy and morphological changes in sensory cells, have emerged as new candidate genes due to their important roles in the development and evolution of echolocation (Li et al. 2007; Mao et al. 2017; Yin et al. 2017). Molecular adaptations contributing to the development of echolocation occur not only in genetic coding sequences but also in the regulation of gene expression (Mao et al. 2017). In species capable of echolocation, certain genes associated with this behavior are expressed at higher levels, and differences in these gene expressions have been shown to contribute to species-specific auditory perception (Shen et al. 2012; Dong et al. 2013). In addition to the molecular characteristics of echolocation, the most comprehensive studies

on the development of this ability and its relationship to cranial features have been conducted in bats. These studies demonstrate that echolocation plays a more determinative role than dietary habits in shaping the morphological diversity of bat skulls (Arbour et al. 2019). Researchers have emphasized that the use of acoustic signals by other echolocating vertebrates (toothed whales, tenrecs, shrews, etc.) and non-echolocating vertebrates can lead to interspecific cranial differences, and that these differences could be further expanded through future research on these species (Jacobs et al. 2014). Non-echolocating bat species display a combination of longer and dorsa-ventrally flattened skulls. Additionally, there are notable changes in certain cranial parameters, such as rostrum length, cheekbone arch length, braincase height, and tympanic bullae size, between skull structure and the use of echolocation (Giacomini et al. 2022). Research on echolocation highlights that understanding similar hearing adaptations among different mammalian species and their relationships will aid in comprehending animal survival strategies, the evolutionary aspects of echolocation, biodiversity, and wildlife conservation (Jones and Holderied 2007; Brinkløv et al. 2022).

This study comprehensively examines the putative adaptation strategies developed by blind mole rats (*Nannospalax xanthodon*) for acoustic communication and navigation, despite the loss of their visual abilities. The research adopts an integrative approach that includes echolocation mechanisms to explore the impact of ecological niches on morphogenetic processes. The study focused on specific genes, which are involved in sound perception and signal processing, and are known to play key roles in the evolutionary modifications observed in echolocating species. Similarly, other genes associated with visual and auditory functions were also included in the study to better understand the sensory characteristics of blind mole rats potentially adapted to subterranean life at the molecular level. The comparative analyses conducted reveal how the distinct molecular and cranial differences between surface dwelling rats (*Rattus rattus*) and blind mole rats reflect ecological niche differentiation. The findings offer new research perspectives for understanding the possible selection pressures exerted by specialized habitats. The study also emphasizes the need for a deeper investigation into the probable underground adaptations, particularly focusing on the neurogenetic basis and its connection to cranial morphology and behavioral ecology. These findings provide an important reference point for sensory evolution and ecological adaptation studies that may be associated with subterranean life.

Materials and methods

Preparation of tissue samples and RNA isolation

RNA isolation was performed using brain tissue samples (20 mg) from a total of 16 animals (8 individuals per species), representing the rodent group (*Nannospalax xanthodon* and *Rattus rattus*), which were stored at -80 °C. The samples belong to wild individuals of *N. xanthodon* with 2n = 60 chromosomes and *R. rattus*, collected from

open fields in the Western Anatolia Region (Bilecik province). The isolation was carried out following the manufacturer's instructions using the innuPREP RNA Mini Kit 2.0 (Analytik Jena GmbH). The collection of these specimens was conducted under permits issued by the General Directorate of Nature Conservation and National Parks, an agency of the Turkish Ministry of Agriculture and Forestry (Permission No: 72784983-488.04), and with approval from the Kırıkkale University Local Ethics Committee for Animal Research (Approval No: 15/02-15-18).

cDNA synthesis

cDNA was synthesized from RNAs using the SCRIPT cDNA Synthesis Kit (Jena Bioscience) according to the manufacturer's instructions. Random primers (0.5 µL from 100 µM stock) were incubated for 5 min at 65 °C for RNA (10 µL from 10 ng/µL stock) binding and then the reaction media were taken on the ice. Then the prepared reaction mixture (Suppl. material 1: table S1) was incubated in a thermal cycler first at 50 °C for 60 min and then at 42 °C for 10 min. After the thermal reaction, the cDNAs were stored at -20 °C.

Real-time PCR analysis

Gene expression analysis was conducted using the LightCycler 480 II system (Roche Diagnostics) with innuMIX qPCR MasterMix SyGreen Sensitive (Analytik Jena GmbH) and 96-well DNase/RNase-free plates. A total of 8 genes were analyzed in this study, including hearing-related genes (*Coch* [Cochlin] and *Necap1* [Adaptin ear-binding coat-associated protein 1]), vision-related genes (*Impg2* [Interphotoreceptor Matrix Proteoglycan 2] and *Pax6* [Paired box 6]), and genes associated with auditory perception (*Kcnq4* [Potassium Voltage-Gated Channel Subfamily Q Member 4], *Foxp2* [Forkhead Box P2], *Tmc1* [Transmembrane Channel Like 1], and *Bmp7* [Bone Morphogenetic Protein 7]) (Parker et al. 2013; Mao et al. 2017; Yin et al. 2017).

Primer design was performed using mRNA sequences from six species: *Nannospalax galili*, *Rattus norvegicus*, *Mus musculus*, *Myotis blythii*, *Myotis myotis*, and *Miniopterus schreibersii*. Conserved regions were identified using the Clustal Omega Multiple Sequence Alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). Conserved regions were then identified by visually examining the alignments to detect stretches of identical nucleotide sequences shared across all species. Suitable regions for primer design were selected based on the following criteria: a length of 19–25 base pairs and identical nucleotide sequences across all species. A total of 23 primer regions were chosen for primer control and Primer-BLAST analysis. Subsequently, the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to screen primers against species from the Rodentia and Chiroptera orders. Factors such as off-target binding, primer dimer formation, and temperature ranges were evaluated to optimize primer suitability before the study. The reagents and gene primers (designed specifically for this study) used in the protocol are provided in Suppl. material 1: table S1 and table S2, respectively.

Univariate statistical analysis of gene expression data

Quantity (relative) values were calculated with the help of the standard graph of Cq (ΔRn) values that changed during gene expression using the LightCycler 480 software. For each species, 8 animals with three replicates, (n = 24) were used in the analysis. The differences in targeted cDNA amount were eliminated by proportioning the targeted gene quantity and reference gene (GAPDH) values in GraphPad Prism 8.0 software. The concentration (ng) of gene expression was normalized with GAPDH (reference gene) expression. The data were analyzed by applying Dunnett's multiple comparisons test with two-way ANOVA. The degrees of significance were denoted as $p \leq 0.05$ *, $p \leq 0.01$ **, and $p \leq 0.001$ ***. The trade-off between data sensitivity and specificity were analysed using ROC (Receiver Operating Characteristic) test in GraphPad Prism 8.0 software.

Sequence alignment, phylogenetic analysis and detection of amino acid convergence

Coding sequences of associated with auditory perception genes identified in the literature as key genes for echolocation, were collected from GenBank (https://www.ncbi.nlm.nih.gov/) and Ensembl Genome Database (https://www.ncbi.nlm.nih.gov/). Echolocating (marked with red) and non-echolocating species (marked with black) were selected based on previous studies, with reference genome for blind mole rats *N. galili* (marked with blue) included as the focal species (Shen et al. 2012; Chai et al. 2020; He et al. 2021). All taxonomy and sequence accession details are provided in Suppl. material 2. Sequence alignments were performed using the MUSCLE (Edgar 2004) algorithm in MEGA X (Kumar et al. 2018). Based on multiple sequence alignments, amino acid substitutions shared between *N. galili* and echolocating bats and whales were identified and analyzed to assess potential functional similarities. Maximum likelihood (ML) phylogenetic trees for the echolocation genes were reconstructed using MEGA X based on nucleotide alignments, and the best-fit substitution models were selected using the Bayesian Information Criterion (BIC) (Felsenstein 1981). The node support was assessed with 1000 bootstrap replicates (Felsenstein 1985). Branch lengths are represented as the number of substitutions per site.

Positive selection and diversifying signals

To detect signals of positive selection, the codeml module of the PAML software was used. The analysis was carried out using nucleotide sequence alignments and a corresponding phylogenetic tree that included *N. galili* as well as echolocating and non-echolocating species (Yang 2007). Several site models were applied to test for positive selection at specific codon sites. Model 0 (one-ratio) assumes a single dN/dS (ω) ratio for all branches and codons. Model 1 (NearlyNeutral) allows two categories of codons: one with $\omega < 1$ and one with $\omega = 1$. Model 2 (PositiveSelection) introduces a third category with $\omega > 1$, indicating potential positive selection. Model 7 (beta) constrains

ω values to the range (0, 1), following a beta distribution. Model 8 (beta & ω > 1) extends Model 7 by allowing a proportion of codons to have ω > 1. Likelihood Ratio Tests (LRTs) were performed to compare nested model pairs (M1 vs. M2 and M7 vs. M8) to assess the presence of positive selection. Additionally, Bayes Empirical Bayes (BEB) analysis was applied under the models that allowed ω > 1 (M2 and M8) to identify specific codons under positive selection and estimate their posterior probabilities.

Multivariate statistical analysis of cranial morphometry

The study analyzed the skulls of 28 adult male blind mole rats (*N. xanthodon*) and 18 adult male rats (*R. rattus*). Selected external cranial parameters associated with sound perception (braincase length, braincase height, greatest skull length, greatest skull height, rostrum length, tympanic bulla length, tympanic bulla width, zygomatic arch length) were measured to 0.01 mm precision using digital calipers. For Principal Component Analysis (PCA), data from 7 cranial parameters were used, with the values being normalized to the greatest skull length to determine the relationship between the skulls of blind mole rats and rat populations. All cranial data were transferred to Unscrambler X 10.3 (Camo, NO) multivariate analysis (MVA) software. The PCA model was generated using the Singular Value Decomposition (SVD) algorithm, and the results were presented as scores and loadings plots. Hotelling's T^2 statistics with a 0.5% confidence limit were applied to identify potential outliers in the PCA scores plot, ensuring that the main variations captured by the model represent the underlying data structure rather than anomalous observations. The loadings plot allowed the analysis of all cranial parameters, as they show the positions that contribute the most to the variation explained by the PC terms. The magnitude of the difference between animals is represented by the loadings or absolute values at a specific position.

Results

Differences in sensory gene expression profiles associated with subterranean life

The expression levels of genes related to vision, such as *Pax6* and *Impg*, hearing, such as *Coch* and *Necap1*, and sensory perception of sound (echolocation), such as *Kcnq4*, *Foxp2*, *Tmc1*, and *Bmp7*, were comparatively analyzed in subterranean blind mole rats (*Nannospalax xanthodon*) and surface-dwelling rats (*Rattus rattus*) to identify changes in gene expression associated with subterranean living conditions. Four echolocation-related genes showed significantly higher expression levels in mole rats (N) compared to rats (R), while genes related to hearing and vision exhibited lower expression levels. In surface dwelling rats, the expression of echolocation-related genes was significantly lower than that of vision and hearing genes (Figs 1, 2).

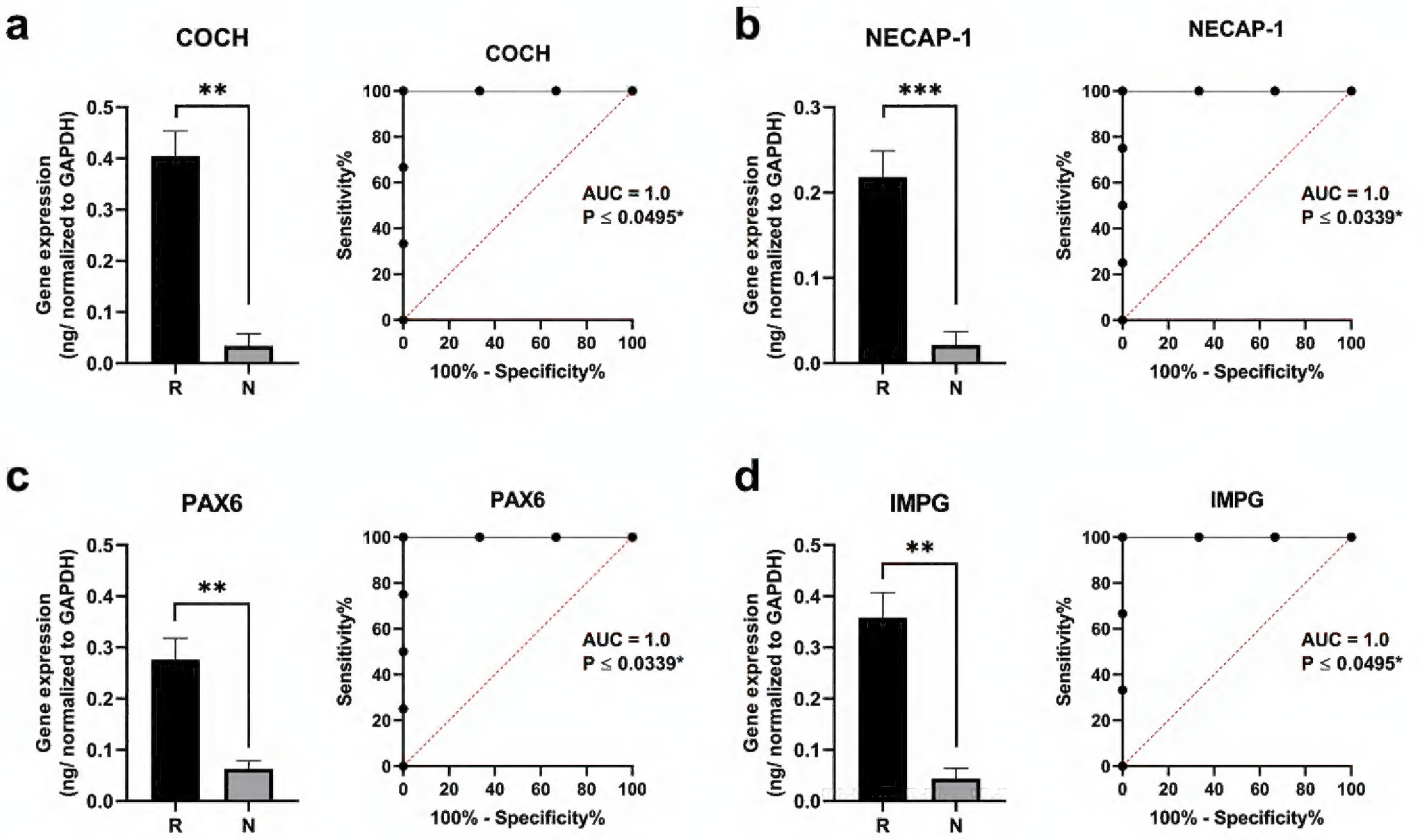

Figure 1. mRNA expression levels of genes associated with hearing function (**a, b**) and vision function (**c, d**) in the brain tissues of *Rattus rattus* (R) and *Nannospalax xanthodon* (N).

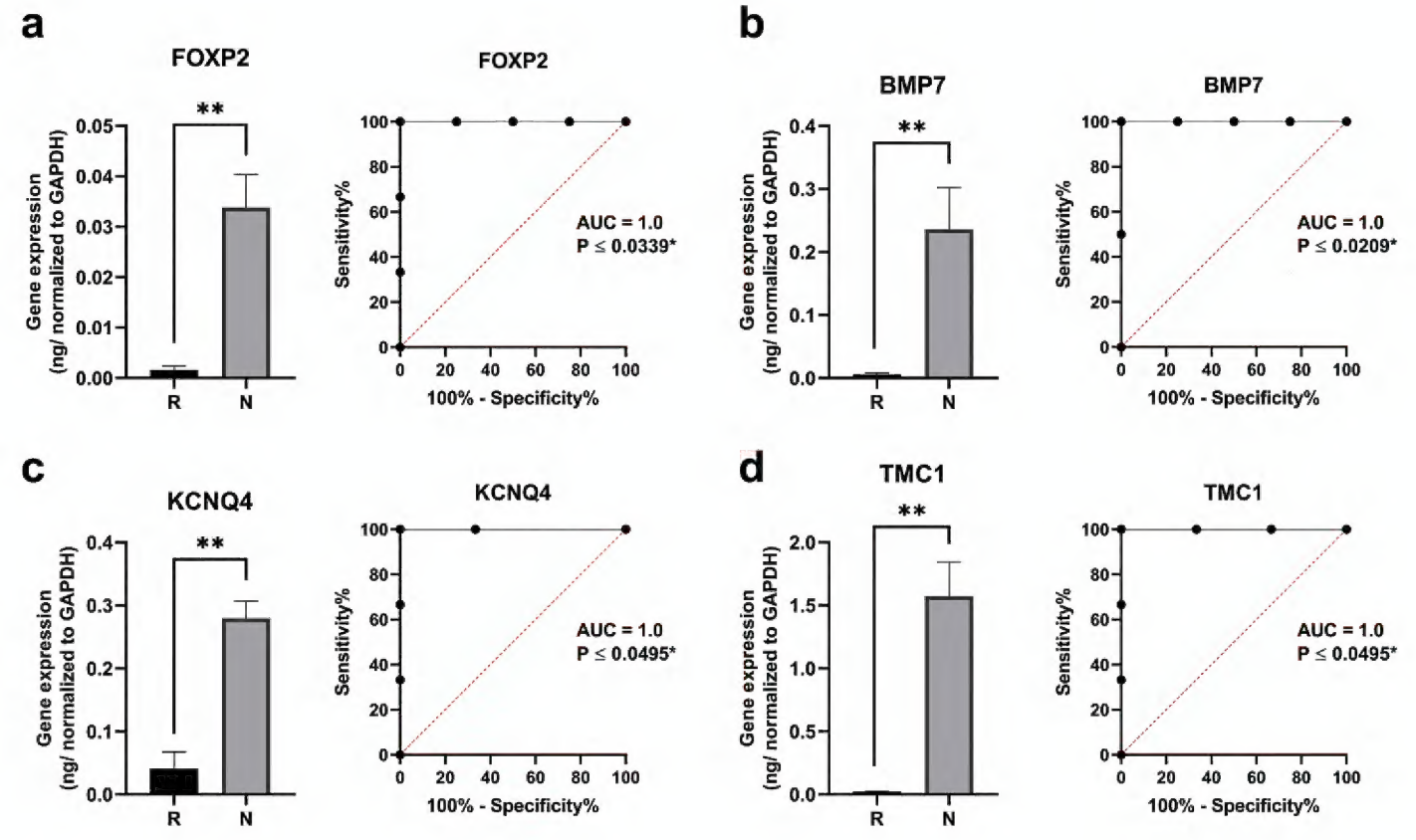

Figure 2. mRNA expression levels of genes associated with echolocation (**a–d**) in the brain tissues of *Rattus rattus* (R) and *Nannospalax xanthodon* (N).

Identification of convergent changes in echolocation-related genes in blind mole rats

To investigate potential convergent evolution related to echolocation, we analyzed six key echolocation genes (*Kcnq4*, *Prestin*, *Bmp7*, *Pjvk*, *Otof*, and *Cdh23*) in *Nannospalax galili* as well as in echolocating and non-echolocating mammals (Parker et al. 2013). Following multiple sequence alignments, we identified nonsynonymous amino acid substitutions particularly shared between *N. galili* and echolocating species (Fig. 3). However, phylogenetic reconstructions based on nucleotide sequences did not cluster *N. galili* within a shared topology alongside high-frequency echolocating species (Suppl. material 1: figs S1–S3).

Sequence alignment of the *Kcnq4* gene across 25 species revealed that *N. galili* shares amino acid substitutions in three different regions with echolocating species (Suppl. material 1: fig. S4A). At position 514, *N. galili* exhibits an S substitution, differing from the C observed in other rodent species, and aligns with echolocating groups such as Yinpterochiroptera and Yangochiroptera. At position 594, the A residue common in rodents is replaced by T in *N. galili*, a change also shared with echolocating Yinpterochiroptera and Yangochiroptera species. Similarly, at position 604, the P residue in rodents is substituted by L in *N. galili*, a modification consistent with echolocating Yinpterochiroptera species. Sequence alignments of the *Prestin* gene across 34 species reveal that *N. galili* shares amino acid changes in three distinct regions with echolocating species (Suppl. material 1: fig. S4B). At position 34, the V residue typical of rodents is replaced by T in *N. galili*, a change also observed in echolocating Yangochiroptera species. At the D611G position, *N. galili* exhibits the same amino acid substitution as *Rihinoplohus luctus.* Also, at the T725L position, *N.galili* shares the same amino acid changes with various echolocating species. Sequence alignments of the *Cdh23* gene from 22 species revealed that this gene shares amino acid changes in five distinct regions with other echolocating species in *N. galili* (Suppl. material 1: fig. S5A). The first change, N502S, is shared with the toothed whale *Lipotes vexillifer* and echolocating Yinpterochiroptera species. Other positions, V864I, R1423Q, F2042I, and finally E2144D, show that *N. galili* shares the same changes with certain echolocating bat species. For the *Otof* gene, alignment of sequences from 24 species, revealed that *N. galili* shares amino acid changes in two regions with echolocating species (Suppl. material 1: fig. S5B). At position R150Q, *N. galili* exhibits the same substitution as the toothed whale *Tursiops truncatus*, while at position G261R, it shares the change with *Physeter macrocephalus*. No shared amino acid changes were identified between *N. galili* and echolocating species in the *Bmp7* and *Pjvk* genes.

PAML site model analyses were conducted to examine the molecular evolutionary patterns of six hearing-related genes (*Kcnq4*, *Prestin*, *Otof*, *Cdh23*, *Bmp7*, and *Pjvk*) across mammalian species (Suppl. material 1: tables S3–S8). The global ω (dN/dS) values estimated under the M0 model were consistently < 1 for all genes (ranging from 0.017 to 0.115), indicating that, overall, these genes are subject to strong purifying selection. To assess the potential for site-specific positive selection, we performed LRTs comparing site models M1 (neutral) vs. M2 (selection) and M7 (beta) vs. M8 (beta

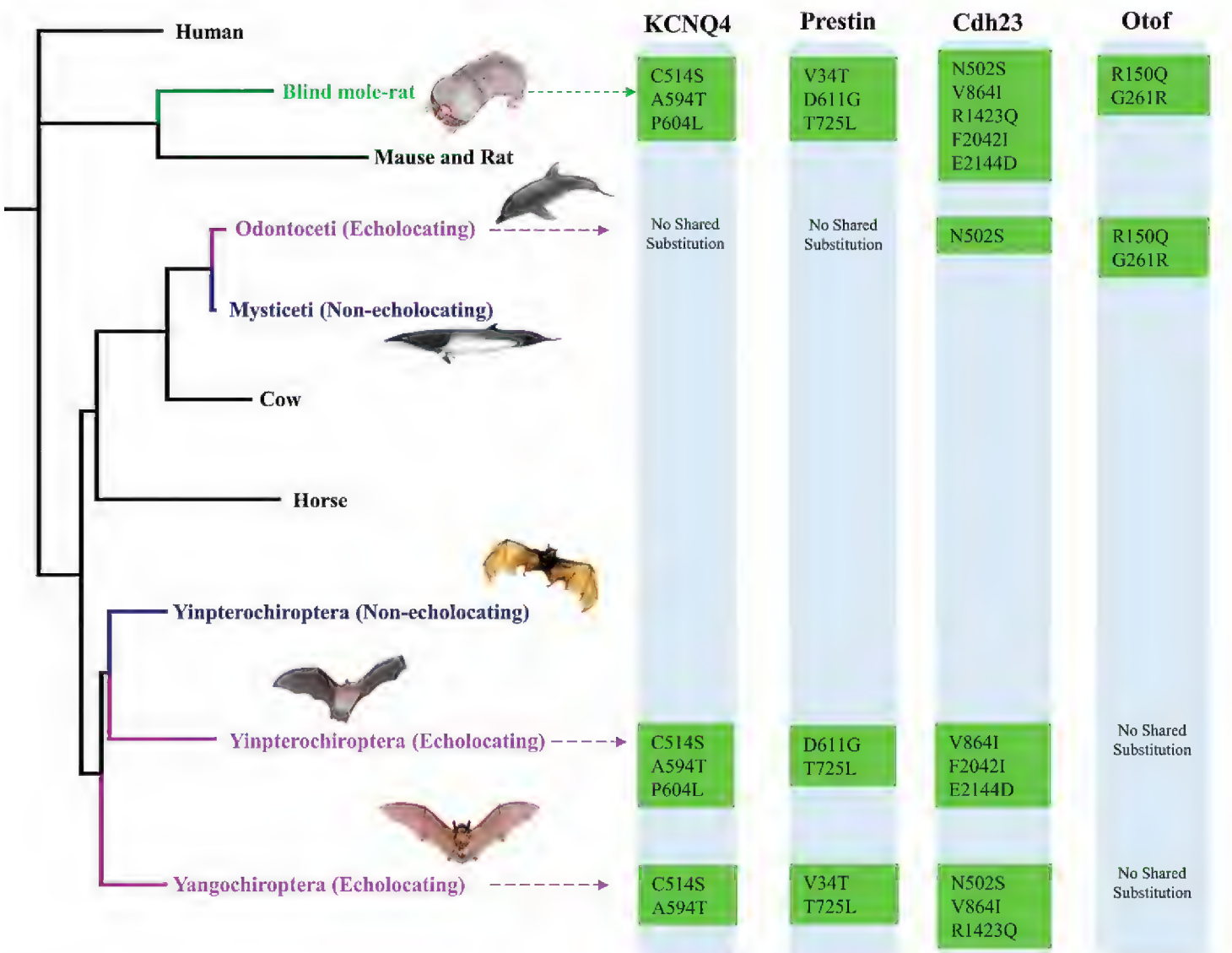

Figure 3. Phylogenetic relationships and amino acid substitutions in auditory sensory perception-related genes among selected mammalian species, including echolocating lineages and the blind mole rat. Species capable of echolocation are highlighted in magenta, while the blind mole rat lineage is shown in green. For each species, amino acid substitutions in four key auditory sensory perception-related proteins (KCNQ4, Prestin, Cdh23, and Otof) are indicated. Substitutions observed in the blind mole rat , and those that are shared between the blind mole rat and at least one echolocating species are highlighted with a green background. (The phylogenetic tree shown in the figure represents an illustrative summary of the ML tree based on *Kcnq4* gene sequences (see Suppl. material 1: fig. S1A for the full tree with bootstrap values and branch lengths)).

& $\omega > 1$). Statistically significant support for the M8 model over M7 was found for five genes: *Kcnq4* ($2\Delta\text{lnL} = 8.37$, $p < 0.05$), *Prestin* ($2\Delta\text{lnL} = 23.71$, $p < 0.05$), *Otof* ($2\Delta\text{lnL} = 16.61$, $p < 0.05$), *Cdh23* ($2\Delta\text{lnL} = 9.72$, $p < 0.05$), and *Pjvk* (M1 vs. M2: $2\Delta\text{lnL} = 8.34$, $p < 0.05$). No significant signal of positive selection was detected for *Bmp7* ($2\Delta\text{lnL} < 0$). Moreover, BEB analysis under model M8 further identified codon sites with high posterior probabilities of $\omega > 1$, especially in *Prestin*, *Cdh23*, and *Kcnq4*, suggesting that these genes may have experienced episodic positive selection at specific sites, despite an overall pattern of purifying selection (Suppl. material 1: tables S3, S4, S6). Notably, among the positively selected sites in *Kcnq4* protein, the substitution at position 443T (corresponding to position 594 in the full alignment of *Kcnq4* gene with all 25 species) is shared between *N. galili* and multiple echolocating mammals (Suppl. material 1: tables S3, S9, fig. S4A). Although several parallel amino acid changes were observed between *N. galili* and echolocating mammals across other sites and genes,

only position 594 (P > 95%) showed a statistically significant signature of positive selection. This implies that not all convergent substitutions are adaptive, and highlights position 594 as the possible candidate for functional convergence under selection.

Comparative morphometric analysis of cranial adaptations for sound perception in subterranean life

The morphometric data were normalized to greatest skull length and analyzed using PCA to statistically evaluate cranial differences and thereby possible auditory adaptations between the species. Morphometric analyses have revealed that the cranial features of *N. xanthodon*, representing a subterranean lifestyle, are significantly distinct from those of *R. rattus* based on characters functionally linked to auditory perception. The most prominent morphological differences between the species, according to PCA analysis, were found in Zygomatic arch length (0.66), Braincase width (0.50), and Rostrum length (0.42) (Fig. 4). These cranial differences are important biological indicators of how the two species have adapted to their ecological environments.

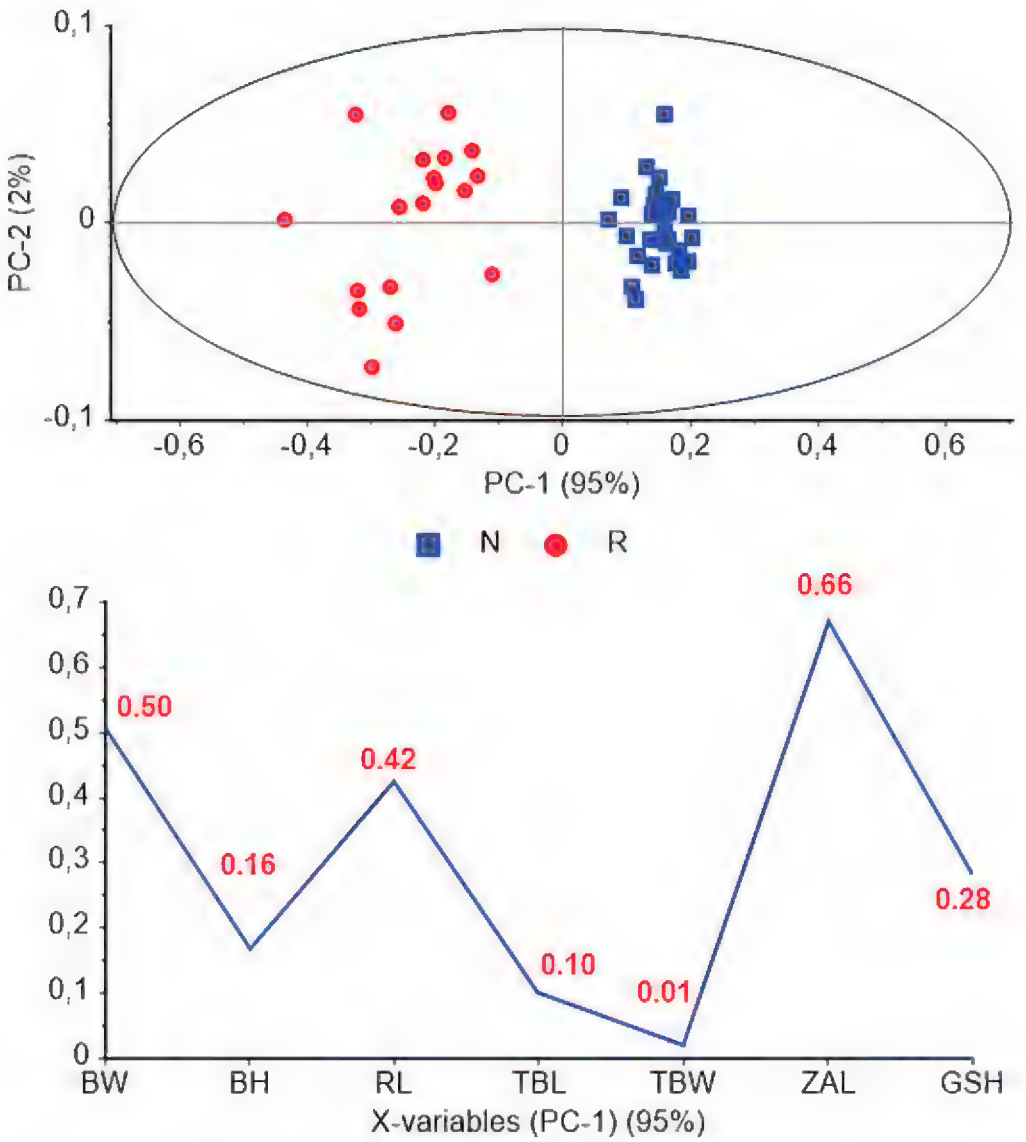

Figure 4. Comparative analysis of external cranial measurements between *Nannospalax xanthodon* (N) and *Rattus rattus* (R). Measured parameters: BW: Braincase length, BH: Braincase height, RL: Rostrum length, TBL: Tympanic bulla length, TBW: Tympanic bulla width, ZAL: Zygomatic arch length, GSH: Greatest skull height.

Discussion

Subterranean life is not confined solely to caves and other natural underground habitats, such as interstitial spaces, but has expanded to include a variety of underground environments with diverse morphologies and microenvironmental conditions. These include shallow subterranean habitats located near the surface (less than 10 meters deep). The defining feature common to all of these environments is the absence of light (Culver and Pipan 2019). The soil tunnel systems constructed by mole rats differ structurally from traditional subterranean habitats such as naturally formed underground cavities. Although these tunnels are excavated by the animals themselves at depths ranging from 20 to 120 centimeters below the surface, they largely exhibit the characteristic environmental conditions of subterranean life, including complete darkness, stable temperature, low oxygen levels, and elevated concentrations of carbon dioxide (Heth 1989; Shams et al. 2005). In species adapted to subterranean biomes, convergent phenotypic changes have occurred in response to similar environmental stressors (Nevo 1999; Leys et al. 2003). In this study, gene expression analyses related to vision, hearing, and echolocation were conducted in *Nannospalax xanthodon*. Additionally, putative evolutionary adaptations associated with subterranean sensory perception were investigated in *Nannospalax galili*, representing blind mole rats, using a comprehensive approach that included positive selection analyses, nonsynonymous site comparisons, and phylogenetic reconstructions within the context of echolocating and non-echolocating mammals. The findings suggest that in *N. xanthodon*, possible subterranean life adaptation is associated with a pronounced negative correlation between classical sensory systems (vision and hearing) and alternative sensory mechanisms (seismic echolocation). This inverse relationship suggests that the increase in echolocation ability to possibly adapt to the special conditions of subterranean life is balanced by a decrease in visual and auditory functions.

Emerling and Springer (2014), investigated a total of 19 genes, including *Impg2*, which are implicated in retinal diseases, in subterranean species (Emerling and Springer 2014). They found that the reduction in the amount of light reaching the retina is associated with an increased regression of retinal genes. Partha et al. (2017), conducted a transcriptional analysis of genes involved in visual pathways in subterranean species such as *Condylura cristata*, *Chrysochloris asiatica*, *Heterocephalus glaber*, and *N. galili* (Partha et al. 2017). This study demonstrated convergent evolutionary rates of visual genes in subterranean habitats and the accelerated convergence of *Pax6* in subterranean mammals, supporting previous findings on visual regression. Functionally inactive visual genes are typically associated with species living in low-light environments (aquatic/subterranean/nocturnal), and it is believed that these genes have lost their functionality, impacting the visual system (Wilkens 2007; Zhao et al. 2009).

We compared the expression levels of certain genes implicated in the development of vision, hearing, and echolocation between blind mole rats (*N. xanthodon*) and rats (*R. rattus*). Our findings revealed that the expression levels of *Pax6* and *Impg* genes, associated with vision, were significantly lower in the subterranean blind mole rats compared to surface-dwelling rats, confirming the regression of light-dependent retinal genes (Fig. 1).

Additionally, the expression differences in vision-related genes involved in the structural development of the eye, combined with the factor of darkness, support the formation of structural differences such as degenerative subcutaneous small eyes in blind mole rats.

In blind mole rats, degenerations such as weak auditory sensitivity, high-frequency hearing loss, and the inability to localize short sounds are characteristics specific to subterranean mammals. It has been suggested that adaptations to subterranean living conditions such as the poor propagation of airborne sounds and the restricted directional movement within tunnels may lead to the degeneration of auditory abilities, similar to how the absence of light diminishes visual capabilities (Heffner and Heffner 1992). *Coch*, associated with hearing or deafness, and *Necap1*, which is expressed in sensory receptor hair cells and processes mechanical inputs to stimulate sensory neurons, have been studied as reference genes in research related to acoustic overstimulation. The absence of the *Coch* gene in noise-exposed mice has been shown to have a protective effect on noise-induced hearing thresholds (Parker et al. 2013; Hickox et al. 2017; Harper et al. 2020; Verdoodt et al. 2021). Accordingly, in our study, it is not surprising that the expression levels of *Coch* and *Necap1*, genes related to hearing, were lower in comparison to surface-dwelling rats, alongside vision-related genes (Fig. 1). However, the decline in the expression levels of these genes may not necessarily indicate a regression of auditory sense; instead, it can be explained as an adaptive regulation in response to the acoustic effects of subterranean tunnels, which function similarly to a stethoscope by amplifying and filtering low-frequency ground vibrations.

On the other hand, the significantly higher expression of *Foxp2*, *Bmp7*, *Kcnq4*, and *Tmc1* genes in blind mole rats compared to rats is intriguing, as these genes play a crucial role in the development of echolocation in echolocating species (Fig. 2). This finding raises the possibility that the regressing sensory functions in blind mole rats may be counterbalanced by the development of echolocation abilities, similar to those observed in bats. Among these genes, *Foxp2* is associated with speech and language in humans and motor dysfunction in mice, and it is linked to acoustic communication in vertebrates and, particularly, the evolution of echolocation in bats (Campbell et al. 2009). The contribution of this gene to sensorimotor coordination in mammals and the significant demands of echolocating bat species for such coordination is being investigated as the primary reason for the functionality of *Foxp2* in all echolocating bat species (Li et al. 2007).

In adult mice, *Bmp7*, which is associated with the cochlea's ability to repair and regenerate lost sensory hair cells, has been examined as a marker gene for inner ear cells. This capability has been attributed to the lower expression levels of this gene postnatally (Lou et al. 2014). In contrast, adult bats exhibit higher expression of this gene compared to younger individuals. The relationship between the *Bmp7* gene and tonotopy, the spatial mapping and perception of complex sound frequencies has been evaluated in adult bats through its higher expression levels, suggesting that it may play a crucial role in the development of echolocation processes (Mann et al. 2014; Mao et al. 2017). It has been reported that the tonotopic organization of the cochlea plays a critical role in the discrimination of acoustic frequencies. Similar to echolocating bats, the tonotopic map of the cochlea in the subterranean species African mole-rat

(*Cryptomys hottentotus*) has been found to contain discontinuous regions with abrupt slope changes (Fettiplace 2023). In this study, the high expression levels of *Bmp7* in mole rats, compared to its low expression in rats, show similarities with the expression profile observed in bats.

In mouse models exposed to high and low-frequency acoustic noise, loss of *Kcnq4* in outer hair cells (OHCs) has been observed, revealing that *Kcnq4* protects OHCs from noise-induced Ca^{2+} overload. Mutations and reduced activity in *Kcnq4* are believed to underlie the common molecular basis of deafness and noise-induced hearing loss. Therefore, the modulation of *Kcnq4* activity is considered an effective strategy for the treatment and prevention of these conditions (Rim et al. 2021). Additionally, the *Kcnq4* protein, localized at the tips of mechanoreceptors, is necessary for the accurate detection of low-frequency signals. Loss of *Kcnq4* has been shown to lead to the degeneration of sensory hair cells, resulting in deafness, while paradoxically increasing sensitivity to low-frequency vibrations (Heidenreich et al. 2012). The significantly higher expression of *Kcnq4* in subterranean, low-frequency sound-guided blind mole rats compared to rats suggests that this gene may play a protective role against low-frequency acoustic noise characteristic of the underground environment.

Tmc1, which is essential for sensory transduction in auditory and vestibular hair cells, has been shown to have therapeutic effects on a variety of auditory disorders, including the behavioral modulation of auditory function in mice through gene therapy applications (Nist-Lund et al. 2019). Among the genes that protect cochlear hair cells from intense sounds in echolocating bats, *Cdh23*, *Kcnq4*, and *Tmc1* are overexpressed in echolocating bats, with protective effects against high noise levels in the cochlear hair cells. A possible link between the protective effects of cochlear hair cells and echolocation has also been suggested (Liu et al. 2021). Gene expression is an important mechanism for possible adaptation or acclimatization to new environments (Charbonnel et al. 2020). In this study, the overexpression of genes associated with echolocation in blind mole rats compared to rats suggests that putative ecological adaptations may contribute to the development of sound localization abilities in subterranean acoustics, similar to echolocating species. However, comparative studies on the expression of genes related to sensory hearing are absent in blind mole rats and are quite limited in rats. This highlights the need for a broader perspective in the investigation of this subject. Therefore, addressing the current gaps in knowledge and providing clearer and more comprehensive explanations regarding how high expression levels of echolocation-related genes contribute to spatial orientation in blind mole rats requires further species-based and tissue-specific research. Notably, studies on the plateau zokor (*Eospalax baileyi*) have revealed that *Prestin*, a gene critical for echolocation, is expressed not only in the cochlea but also in the tail, footpads, and rhinarium. These findings suggest that this gene may play a potential role in detecting low-frequency seismic signals and facilitating spatial orientation in subterranean environments (Dong et al. 2013; Lina et al. 2016). Furthermore, in recent years, the existence of sensory modality trade-offs between vision, hearing, and echolocation in bats has been identified. It has been emphasized that these trade-offs are associated with ecological specialization and that this evolutionary hypothesis requires further

investigation across phylogenetically distinct taxa (Speakman 2008; Zhao et al. 2009; Thiagavel et al. 2018; Wu et al. 2018). The findings of our current study demonstrate that gene expression levels in blind mole rats support this hypothesis, suggesting that similar evolutionary mechanisms may exist in subterranean mammals.

In some cases, it is thought that selection may act on both coding sequences and gene regulation to provide enhanced hearing in echolocating mammals (Mao et al. 2017). Although bats and dolphins, which possess echolocation abilities, use different organs to produce and emit sound, seven genes (*Cdh23*, *Kcnq4*, *Otof*, *Pcdh15*, *Pjvk*, *Prestin*, *Tmc1*) associated with auditory function represent a convergent genetic evolutionary model that is key for echolocating species (Parker et al. 2013). While some genes showing adaptive sequence convergence have been identified between bats and dolphins, *Pcdh15* gene does not support this hypothesis. *Otof* exhibits minimal convergence in dolphins and Yinpterochiroptera, whereas *Cdh23* shows high convergence between Yinpterochiroptera and cetaceans, as well as among some members of Yangochiroptera (Lambert et al. 2017). Echolocation in species other than whales or bats (such as some echolocator bat species, shrews, swiftlets, oilbirds, and even blind humans) is described as simple or passive. This is because they can perform echolocation for spatial orientation without relying on ultrasonic (>20 kHz) sounds (Brinkløv et al. 2022). In these species, evolutionary changes in genes involved in echolocation are less pronounced compared to echolocating bats. However, convergent signals have been detected in some hearing-related genes, such as the *Tmc1* gene in oilbirds (Sadandan et al. 2023). Analyses of hearing-related genes have revealed convergent amino acid changes between the common shrew and other echolocating mammals (bats and dolphins), with 7 changes in *Cdh23*, 5 in *Otof*, and 1 in *Prestin* genes (Chai et al. 2020). The echolocation ability of soft-furred tree mice (*Typhlomys*) has been molecularly supported by the detection of 12 convergent amino acid changes in 217 hearing genes within an echolocating clade (He et al. 2021; Jones 2021).

Common adaptive strategies in response to similar ecological niches are widespread among mammals and represent significant evolutionary traits that ensure their survival. Mammals, such as the bottlenose dolphin (*Tursiops truncatus*), common shrew (*Sorex araneus*), subterranean naked mole rat (*Heterocephalus glaber*), and some bat species, exhibit convergent genomic changes, including losses of antioxidant genes (e.g., *GPX6*), as adaptations to oxidative stress environments (Tian et al. 2021). Subterranean mammals, including blind mole rats (*N. galili*), adapt to hypoxic and hypercapnic conditions in their underground habitats, leading to higher expression of hypoxia-related genes compared to surface-dwelling rats. High carbon dioxide adaptation is demonstrated not only through gene expression but also via genomic analyses, revealing convergent evolution with similar amino acid changes in subterranean mammals (*N. galili*) and bats (*Myotis lucifugus*) (Fang et al. 2014b). Shared nonsynonymous amino acid changes were identified at 3 sites in *Kcnq4* (in Yinpterochiroptera and Yangochiroptera), 3 sites in *Prestin* (in Yinpterochiroptera and Yangochiroptera), 5 sites in *Cdh23* (in the toothed whale *Lipotes vexillifer*, Yinpterochiroptera, and Yangochiroptera), and 2 sites in *Otof* (in the bottlenose dolphin *Tursiops truncatus* and the sperm

whale *Physeter macrocephalus*) (Fig. 3). No shared amino acid changes were detected in the *Bmp7* and *Pjvk* genes between *N. galili* and echolocating species. However, according to the PAML site model analysis of the six auditory genes (*Kcnq4*, *Prestin*, *Otof*, *Cdh23*, *Bmp7*, and *Pjvk*) examined in this study, most of the tested genes were under strong purifying selection. Among these genes, *Kcnq4* showed a positive selection signal at a shared site between *N. galili* and echolocating species. However, phylogenetic trees based on all six genes did not cluster *N. galili* within a combined phylogenetic topology with echolocating species. The genetic profile of blind mole rats, which reflects putative auditory adaptations suitable for subterranean life, does not directly relate to ultrasonic echolocation but suggests that possible adaptations in certain genes could support the development of low-frequency hearing, which may enhance acoustic communication and navigation in underground environments.

Blind mole rats (*Nannospalax ehrenbergi*) use self-generated seismic waves as an echolocation mechanism to determine the size and shape of obstacles. Other subterranean species that produce seismic signals by striking their heads, hind legs, or incisors include the African mole rat (*Georychus capensis*), Damaraland mole rat (*Fukomys damarensis*), giant mole rat (Fukomys mechowii), and demon African mole rat (*Tachyoryctes daemon*) (Hrouzková et al. 2013). One of the methods used for receiving seismic signals in subterranean rodents is the transmission of auditory signals either directly through air conduction via the auditory nerve or indirectly through bone conduction, such as the jaw or skull. Bone conduction can be an effective signal transmission pathway in subterranean rodents, and some species use this method for seismic communication (Dong et al. 2013). Vibrational auditory stimuli reach the inner ear through bone conduction via skull structures. This mechanism in blind mole rats, known as "hearing through the jaw," has been confirmed in *Tursiops truncatus* and some digging reptiles where mandibular adipose tissue transmits acoustic signals (Rado et al. 1989). Some structures of the skull, such as enlarged auditory bullae, have potential advantages for better seismic detection and are common in fossorial rodents due to the slower attenuation of low-frequency sounds in underground tunnels (Hennekam et al. 2021).

The cranial features of *N. xanthodon*, adapted to subterranean life, significantly differ from *R. rattus* in terms of auditory-related parameters. The most notable differences were observed in the measurements of zygomatic arch length (0.66), braincase width (0.50), and rostrum length (0.42) (Fig. 4). The wide zygomatic arches, characteristic of fossorial mammals, are generally associated with strong fossorial adaptations (Gomes Rodrigues et al. 2016). In blind mole rats, the seismic signal detection mechanism via bone conduction primarily occurs through the effective transmission of vibrations from the lower jaw and cheek region to the *incus* and *stapes* (Rado et al. 1998; Hill et al. 2019). Bone conduction is more effective than air conduction in transmitting low-frequency sounds to the cochlea and has a more pronounced impact on the nervous system (Joshi and Bendale 2020). The fine and wide zygomatic bone structure in *N. xanthodon* may play a significant role in detecting sound waves from the surface of the soil, along with digging behavior. Recent studies have highlighted the critical role of the zygomatic arch in bone conduction and shown that hearing thresholds in the facial region exhibit

characteristics similar to those of the mastoid process (Uemura et al. 2022). Another feature proposed to be associated with the processing of seismic communication signals is the skull width observed in *Nannospalax*, golden moles, and some other species (Mason and Narins 2001). Allometric studies have shown that the brain of *Nannospalax* is larger than that of tenrecs and rats (Frahm et al. 1997). Skull width and braincase width, in particular, emerge as important parameters in studies focused on the echolocation abilities of bats (Giacomini et al. 2022). The size of the braincase has been explained by the fact that bats have a sufficiently designed auditory brain structure to enable echolocation, despite their reduced eyes (Thiagavel et al. 2018). Regarding the processing of sensory modalities, it has been noted that extensive areas of the occipital cortex in blind mole rats are activated by auditory stimuli, and similar comprehensive studies suggest that the lower regions of the auditory cortex are dedicated to processing the sounds they emit and the echoes used for echolocation, as seen in echolocation bats (Sadka and Wollberg 2004). Finally, rostrum length is generally known as a parameter associated with bite force and diet in mammals. However, in subterranean fossorial mammals like *N. xanthodon*, the rostrum, along with the top part of the head, contributes to the formation of sound waves by striking the tunnel walls. Therefore, it can be suggested that rostrum length has a functional impact on the species' sound perception mechanisms. The cranial structure of blind mole rats shows significant flexibility in response to ecological parameters. These morphological differences provide important clues about the species' subterranean lifestyle and acoustic perception abilities. However, the existing literature has generally addressed these putative adaptations in the context of digging behavior (fossorial traits). This study emphasizes that the cranial features of subterranean mammals like *N. xanthodon* are not only limited to possible fossorial adaptations but may also be related to acoustic perception and environmental interactions. These findings highlight the need for more comprehensive research in the cranial domain.

Conclusion

This research suggests that gene expressions related to sensory communication in blind mole-rats may play a role in seismic vibration detection, a distinct variant of echolocation possibly adapted to subterranean life. While their visual functions have diminished, the decline in auditory functions may indicate their potential adaptations to the unique acoustic environment underground. However, significant gaps remain in our understanding of the mechanisms underlying seismic echolocation and its evolutionary trajectory. Future studies should focus on expanding the scope of the investigation to include a broader range of subterranean species, which will allow for comparative analyses and the identification of shared or divergent genetic adaptations. Additionally, tissue-specific investigations are crucial for pinpointing the precise roles of candidate genes in sensory perception and navigation. Beyond the genes associated with ultrasonic echolocation, there is a need to explore novel genetic factors that may support the development of alternative echolocation strategies tailored to subterranean environments. Such research

not only holds the potential to uncover fundamental insights into the molecular basis of sensory evolution but also offers broader implications for understanding convergent evolution and ecological adaptation that may be associated with subterranean life. The structure of the skull may show potential adaptations that enhance the effectiveness of bone conduction, which is necessary for this type of acoustic transmission. However, it is important to note that the same anatomical features are also heavily influenced by digging behavior. Therefore, more comprehensive and complex analyses supported by a greater number of subterranean species are required to disentangle these two effects. By elucidating the interplay between genetics, environment, and behavior, future studies could provide a more comprehensive framework for examining the diversity of navigation strategies in subterranean habitats. Ultimately, these efforts will offer deep insights into how life adapts to even the most challenging and specialized niches on Earth.

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Competing interests

The authors have declared that no competing interests exists.

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Supplementary material 1

Supplementary figures nad tables

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Supplementary material 2

Supplementary data

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